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Dechlorination of Commercial PCBs and Other Multiple Halogenated Compounds by a Sediment-Free Culture Containing Dehalococcoides and Dehalobacter

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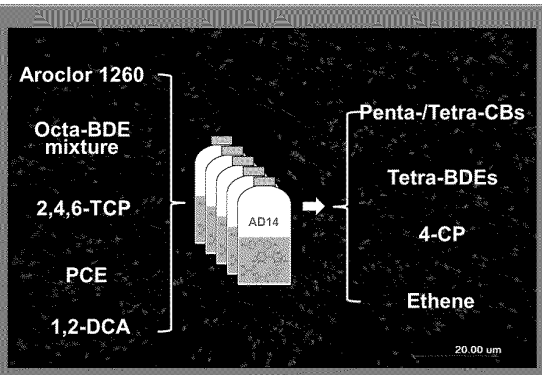
Supporting Information

At the contaminated sites, polychlorinated biphenyls (PCBs) frequently coexist with other halogenated compounds, such as polybrominated diphenyl ethers (PBDEs), chloroethanes, and chloroethenes. The presence of multiple halogenated compounds usually poses toxicity to dehalogenating microbes, because few cultures are capable of detoxifying a broad spectrum of halogenated compounds. In this study, a sediment-free culture, designed as AD14, is able to sequentially remove halogens from PCBs and other cocontaminants. Culture AD14 dechlorinated the commercial PCB mixture • Aroclor 1260 • mainly by removing flanked para- and doubly flanked meta-chlorines. It also dehalogenated octa-brominated diphenyl ether mixture predominantly to tetra-BDEs, 2,4,6-trichlorophenol (2,4,6-TCP) to 4-CP, and tetrachloroethene (PCE)/1,2-dichloroethane (1,2-DCA) completely to ethene. When applied to a mixture of the above-mentioned compounds, culture AD14 stepwise removed halogens from 2,4,6-TCP, 1,2-DCA, PCE, PBDEs, and PCBs. Illumina sequencing analysis of 16S rRNA genes showed that only two known dechlorinating genera, Dehalococcoides and Dehalobacter, were present in culture AD14. Quantitative real-time PCR analysis showed that the 16S rRNA gene copies of Dehalococcoides and Dehalobacter increased from 1.14×10^5 to 7.04×10^6 copies mL^{-1} and from 1.15×10^5 to 8.20×10^6 copies mL^{-1} after removing 41.13 μM of total chlorine from PCBs. The above results suggest that both Dehalobacter and Dehalococcoides could be responsible for PCB dechlorination. Although two Dehalococcoides mccartyi strains with identical 16S rRNA genes were isolated from the PCBs-dechlorinating mixed culture using trichloroethene (TCE) and vinyl chloride (VC) as alternatives to PCBs, the two isolates are incapable of dechlorinating PCBs. In all, culture AD14 is promising for bioremediation applications at sites cocontaminated with PCBs and other halogenated compounds.



INTRODUCTION

Polychlorinated biphenyls (PCBs) are a family of 209 congeners that were produced and sold as complex mixtures, e.g., Aroclor 1260. Although the production of PCBs has been banned in most countries since the late 1970s, their massive industrial usage has resulted in their widespread distribution in sediments of many lakes, rivers, and harbors.[1] Thus, PCBs still remain a major concern to the health of human beings and ecosystems.[2] For example, an exponential increase in the concentrations of PCBs and polybrominated diphenyl ethers (PBDEs) was found in dolphins and sharks from Florida coastal waters based on a 10-year period study.[3] A more troublesome problem is that many PCB-contaminated sites are cocontaminated by other various halogenated compounds such as PBDEs[4,5] and chloroethenes,[6] posing challenges to the bioremediation strategies.

Thus far, chlorine removal from highly chlorinated PCB congeners has been observed in anaerobic environments via a microbial reductive dechlorination process.[7] Many PCB-dechlorinating cultures have been established with sediments from different geographic sites, in which the PCB dechlorinators were identified to be either Dehalococcoides[7−11] or o-17/DF-1-like Chloroflexi bacteria.[7,10,12] Among them, only three cultures showed the capabilities to dehalogenate both PCBs and other halogenated compounds, i.e., Dehalococcoides mccartyi strain 195, Dehalococcoides mccartyi strain CBDB1 and Dehalobium chlorocoercia DF-1.[8,11,13,14] Strain 195, best known for perchloroethene (PCE) and trichloroethene (TCE) dechlorination, has been reported to dehalogenate three PCB congeners[11] and PBDEs.[13] Strain CBDB1 has broad dechlorination activity on chlorinated aromatic compounds, e.g., chlorobenzenes,[15] chlorinated dioxins,[16] and Aroclor 1260.[8] Bacterium DF-1 can dechlorinate weathered Aroclor 1260 through attacking double-flanked chlorines,[14] and hexachlorobenzene to 1,3,5-trichlorobenzene.[17] However,

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strain 195 is known only to dechlorinate PCB congeners chlorinated on a single ring (i.e., 23456-CB, 2346-CB, and 2356-CB), which are usually not the PCBs present at contaminated sites. Information on PDBE debromination by both strains CBDB1 and DF-1 is not available, and their PCE/TCE dechlorination can only extend to trans- and cis-dichloroethenes (DCEs).[18,19] Therefore, information remains limited on cultures capable of dehalogenating mixtures of PCBs and their frequently coexisting halogenated compounds (e.g., PBDEs and chloroethenes). Furthermore, the effect of the coexistence of other halogenated compounds on PCBs dechlorination is still unknown.

The aim of this work was to cultivate and characterize an enrichment culture AD14 that could extensively dechlorinate Aroclor 1260 and other multiple halogenated compounds; to identify the dechlorinating bacteria by using 16S rRNA gene based Illumina sequencing approach; to further enrich and isolate the dechlorinators by using alternative chlorinated compounds (e.g., chloroethenes).

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, chemicals were purchased from Sigma-Aldrich at the highest purity available. All PCBs were purchased from AccuStandard (New Haven, CT, U.S.). H_2 was obtained from a hydrogen generator (NM-H250, Schmidlin-DBS AG, Neuheim, Switzerland).

Microcosm Preparation, Culture Transferring and Growth Conditions. The slurry used for preparing PCB dechlorinating microcosms was sampled from an anaerobic digester in a wastewater treatment plant in Gehua (Hubei Province, P.R. China), in which concentrations of PCBs, PBDEs, chlorophenols, chloroethenes, and chloroethanes were under detection limit (<0.1 μM). The sample was acquired by filling sterile 50-mL plastic Falcon tubes that were capped and transported to the laboratory at an ambient temperature. Microcosm setup was conducted in anaerobic chamber as previously described.[13,20] Briefly, 90 mL of bicarbonate-buffered mineral salt medium amended with 10 mM of lactate was dispensed into 160 mL serum bottles containing 10 mL of the slurry. The bottles were sealed with black butyl rubber septa (Geo-Microbial Technologies, Ochelata, OK, U.S.) and secured with aluminum crimp caps. Then, a 60 μL of Aroclor 1260 (AccuStandard, New Haven, CT, U.S.) stock solution (50 mg of total PCBs per mL) in GC grade isooctane was spiked into the medium to a final concentration of 30 ppm (or 80.65 μM). The microcosms were incubated stationary in the dark at 30 °C. PCB dechlorination activities were measured frequently with a gas chromatograph equipped with an electron capture detector (GC-ECD), as described in the following section. Sediment-free cultures were obtained by six consecutive supernatant transfers of the active microcosm to the same fresh medium (5%, v/v) as described.[20] Cultures amended with two individual PCB congeners (i.e., 2345-245-CB and 234-245-CB) were also prepared to determine their dechlorination pathways in the sediment-free cultures. The sediment-free PCB dechlorinating culture was used to inoculate (2%, v/v) four subcultures amended with PCE (~0.7 mM), 1,2-DCA (~0.6 mM), 2,4,6-TCP (~50 μM), and octa-BDE mixture (0.4 ppm, dissolved in TCE), respectively. Cultures amended with all these halogenated compounds (~ 15 ppm or 40.32 μM Aroclor 1260, ~0.1 mM PCE, ~0.6 mM 1,2-DCA, ~10 μM 2,4,6-TCP and 0.1 ppm octa-BDE mixture) together were also prepared to study their inhibition effects to PCB dechlorination and their dechlorination priorities. After observing dehalogenation activities in the four subcultures, enrichments through serial transfers were conducted using the same halogenated compound in the media amended with acetate (10 mM)/hydrogen (5×10^4 Pa or 0.40 mM), a vitamin solution consisting of 0.05 mg l^{-1} of vitamin B_{12}, and 2% inocula of respective subcultures to prevent the growth of fermentative bacteria.[13] Since Dehalococcoides resist the antibiotic ampicillin, further enrichment was conducted by serial dilutions in 20 mL vials filled with 10 mL of mineral salts medium spiked with TCE (0.8 mM) or VC (0.4 mM), acetate (10 mM), hydrogen (5×10^4 Pa or 0.40 mM), and ampicillin (50 ppm). Culture purity was confirmed via DGGE, clone library and qPCR analysis. After obtaining pure cultures, dechlorination time-course studies were conducted in triplicate 160-mL serum bottles containing 100 mL of mineral salts medium amended with TCE (~0.8 mM) or VC (~0.4 mM), acetate (10 mM)/hydrogen (5×10^4 Pa or 0.40 mM), a vitamin solution consisting of 0.05 mg L^{-1} of vitamin B_{12}, and 5% inocula. The following compounds were tested on the new isolates as a sole electron acceptor: chlorinated ethenes (DCE isomers and VC) (0.2 mM); 1,2-DCA (0.2 mM), Aroclor1260 (30 ppm), octa-BDE mixture (0.5 μM), or 2,4,6-TCP (50 μM). In addition, the isolates were also tested for their ability to use the following compounds (10 mM each): fumarate, malate, lactate, pyruvate, glucose, glumate, sulfate, sulfite, nitrate, or nitrite. All experiments were set up in triplicate. Duplicate abiotic controls (without bacterial inocula) and non-PCB controls (without PCBs injection) were also set up for each experiment.

Analytical Methods. Headspace samples of chloroethanes, chloroethenes, and ethene were injected manually with a glass, gastight, luer lock syringe (Hamilton, Reno, NV, U.S.) into a gas chromatograph (GC) 6890N equipped with a flame ionization detector (Agilent, Wilmington, DE, U.S.) and a GS-GasPro column (30 m × 0.32 mm × 0.25 μm film thickness; J&W Scientific, Folsom, CA, U.S.). PCBs, PBDEs, and chlorophenols were extracted as described.[21] Before chlorophenols' extraction, derivatization was conducted by taking 1 mL of liquid sample and mixing with 5 mL of potassium carbonate solution (5% w/v), acetylated with 200 uL acetic anhydride. PCBs were measured with the same GC but equipped with an electron capture detector (GC-ECD) and a DB-5 capillary column (30 m × 0.32 mm × 0.25 μm film thickness; J&W Scientific, Folsom, CA, U.S.) as described.[22] The temperature program was initially held at 170 °C for 5 min, increased at 2.5 °C min^{-1} to 260 °C, and held for 10 min. Injector and detector temperature were 250 and 300 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. Sample of one μL was injected into the GC inlet in a splitless mode. The elution time of all 209 PCB congeners was determined with PCB congener mixtures 1 through 9 from AccuStandard. The relative elution time of the PCBs in these mixtures were published for the DB-5 column.[23] PCBs were quantified by using a customized calibration standard prepared from Aroclor 1260 plus 33 congeners that are possible dechlorination products and intermediates.[24] Additional congeners were quantified from standards prepared from the AccuStandard PCB congener mixtures. Mole percent value for each congener, total number of chlorines per biphenyl and the PCB homologue distribution were calculated as described.[25] PBDEs were tested and quantified by gas chromatograph/mass spectrometer (GC-MS) with a model of GC 6890/MSD 5975 apparatus (Agilent, Wilmington, DE,

U.S.) equipped with a Restek Rxi-5 ms column (15 m × 0.25 mm × 0.25 μm film thickness; Restek Corporation, Bellefonte, PA, U.S.) as described.[21] Chlorophenols (2,4,6-TCP, 2,4-DCP and 4-CP) were analyzed on a GC-MS set in a selected ion monitoring mode (QP 2010, Shimadzu Corporation, Japan) and equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm film thickness; J&W Scientific, Folsom, CA, U.S.). The oven temperature of the GC-MS was initially set at 40 °C, increased at 15 °C min^{-1} to 200 °C, and held for 3 min. Helium was used as the carrier gas, with a column flow of 1.92 mL min^{-1}. Derivatized sample of 1 μL was injected into the GC inlet in a splitless mode with 250 °C injector temperature. Chlorophenols were quantified by using customized calibration standards at gradient concentrations from 5, 10, 20, 30, 40, 50, to 60 μM.

DNA Extraction, PCR, Clone Library, And Sequencing. Total genomic DNA was extracted from 1 mL of dehalogenating culture and the control according to the manufacturer's instructions (DNA extraction kits, QIAGEN, Hilden, Germany) but with minor modifications.[26] The concentration of the nucleic acid was determined by a Nanodrop-1000 instrument (NanoDrop Technologies, Wilmington, DE, U.S.). Amplifications of 16S rRNA gene and other gene sequences were conducted with GoldTaq DNA polymerase (Applied Biosystems, Foster City, CA, U.S.) by using a Mastercycler (Eppendorf, Hamburg, Germany) under conditions previously described.[20] The primer sequences used in this study are shown in Table S1 of the Supporting Information, SI. Clone libraries were established by using TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, U.S.), and all further clone-based experiments were carried out as previously described.[26] Purified plasmids or PCR products were sequenced and aligned by using MEGA4.[27]

Illumina High Throughput Sequencing Analysis of 16S rRNA Genes. To analyze the taxonomic composition of the sediment-free PCB dechlorinating culture, the V9 region of the 16S rRNA gene (from base 1392-1509, E.coli numbering) was chosen for PCR amplification with the universal primer set (targeting most of the archaeal 16S rRNA genes) containing a barcode sequence (underlined) - 1392F (5′-ACAGCTCAG-YACACACCGCCCGTC-3′) and 1492R (5′-GGYTACCTT-GTTACGACTT-3′). Amplified PCR products were purified by using QIAquick PCR purification kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions. Then the PCR sample was mixed with other samples for subsequent Illumina high throughput sequencing, of which sequences will be differentiated based on barcode sequences of the forward primers. Illumina (Highseq2000, Illumina, San Diego, CA, U.S.) sequencing services were provided by BGI (Hongkong, P.R. China). Raw sequencing reads were checked for their quality through elimination of sequences that did not perfectly match the proximal PCR primer and that with short sequencing length (<70 nt). A total of 34 724 pair-end reads were obtained for the PCB dechlorinating culture with an average read length of 120 bp. Pair-end reads were joined to form longer composite reads by using the SHERA software package.[28] Sequence alignments were conducted with each subset reads based on NAST,[29] and with other settings kept at their default values as described.[30] After NAST alignment, aligned subsets were merged into one Microsoft Excel file, in which sequences were clustered (based on 97% of sequence similarity) according to template ID. Manual adjustments were performed to improve the alignment and clustering whenever necessary. Representative sequences for each cluster were identified through RDP Classifier with 100% coverage and 95% confidence threshold[31] and BLAST analysis,[32] which were further utilized to construct phylogenetic tree by using MEGA 4.[27]

DGGE. PCR products amplified with the GC-clamped primer sets were separated on an 8% polyacrylamide gel with a gradient range of 30−60% (100% denaturant consisting of 7 M urea and 40% deionized formamide) in 0.5 × TAE buffer. Gradient gels were cast with Bio-Rad's Model 475 gradient delivery system (Bio-Rad, Hercules, CA, U.S.) as described.[33] The electrophoresis was performed for 15 h at a constant electric current of 30 mA and a temperature of 60 °C with the D-Code Mutation Detection System (Bio-Rad, Hercules, CA, U.S.). Gel images of SYBR Gold (Invitrogen, Carlsbad, CA, U.S.) staining DNA were taken by using a Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, U.S.). Bands of interest were excised and DNA fragments were extracted by using the QIAEX II Gel Extraction Kit (QIAGEN, GmbH, Germany). The captured DNAs were then PCR reamplified and reanalyzed by DGGE to confirm that single bands were obtained before sending the PCR reamplified products for sequencing. Two step DGGE (2S-DGGE) was performed as previously described,[33] which was developed to obtain full-length 16S rRNA gene sequences simultaneously from multiple samples.[33]

qPCR. A TaqMan quantitative real-time PCR (qPCR) (ABI 7500 Fast real-time PCR system; ABI, Foster City, CA, U.S.) assay was performed in triplicate for PCB dechlorinating cultures by using Bacteria and Dehalococcoides 16S rRNA gene-targeted and tceA/vcrA gene-targeted primers/probes, respectively, as described previously.[26] Dehalobacter species in these cultures was quantified by targeting 16S rRNA genes using SYBR green assays. The primer and probe sequences used in this study were shown in Table S1 of the SI. A calibration curve was obtained by using 10-fold serial dilutions of known plasmid DNA concentrations. The standard curves spanned a range of 10^2 to 10^8 gene copies per μL of template DNA. Nuclease-free water or plasmid without an insert was used as the negative control.

Nucleotide Sequence Accession Numbers. The nucleotide sequence data obtained in this study were submitted to the Genbank with the following accession numbers: KC342960-KC342971.

RESULTS

Reductive Dechlorination of Aroclor 1260. A sediment-free culture, designed as culture AD14, was obtained after six serial transfers (5% inocula, v/v) in the defined medium amended with Aroclor 1260 (30 ppm or 80.65 μM) and lactate (10 mM). Figure 1 showed the PCB congener distribution in abiotic controls (without inocula) and in cultures AD14 after 120 days of incubation. The abiotic controls showed no obvious difference from original Aroclor 1260. In culture AD14, major hexa- through octa-CB congeners of Aroclor 1260 were substantially dechlorinated to lower halogenated PCB congeners, of which the prominent dechlorination products were penta- (i.e., 245-25-CB, 245-24-CB, 235-25-CB, and 236-24-CB) and tetra-CB congeners (i.e., 25-25-CB, 24-25-CB, and 24-24-CB). Four major hepta-CB congeners, accounting for 59.61 mol % of total hepta-CB congeners, experienced more than 50% decreases, i.e., 2345-245-CB (a 65.61% decrease), 2345-236-CB (55.66%), 2345-234-CB (66.06%), and 2346-245-CB (66.55%). Two most abundant hexa-CB congeners in Aroclor

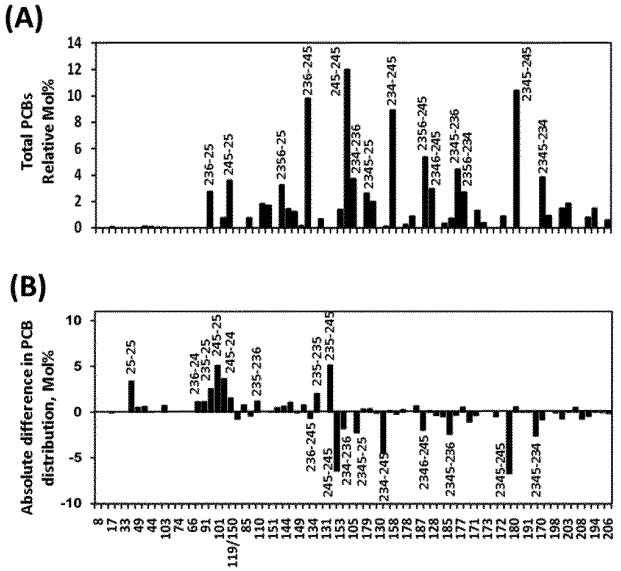

Figure 1. (A) Congener distribution in the abiotic control and (B) differences in congener distribution of Aroclor 1260 residues between the control bottles and culture AD14 after 120 days of incubation. Abiotic controls showed no changes from original Aroclor 1260. The congeners with obvious changes in relative abundance were indicated.

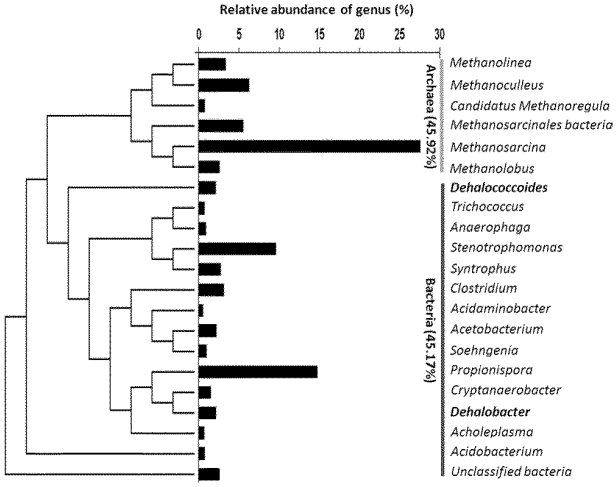

Figure 2. Phylogenetic compositions of culture AD14 (after 120 days of incubation with Aroclor 1260) obtained via Illumina high throughput sequencing of 16S rRNA genes. The percentage of bacteria/archaea from each genus was calculated, and only the genera with ≥0.5% in relative abundance were shown. Known dechlorinators presented in this enrichment culture were highlighted with bold font. Phylogenetic analyses was conducted via neighbor-joining with MEGA4.[27]

1260, 245-245-CB, and 234-245-CB, were significantly reduced from 11.94 mol % to 5.10 mol % (a 57.29% decrease), and from 8.90 mol % to 4.24 mol % (a 52.36% decrease), respectively. However, several other hexa-CB congeners (e.g., 235-245-CB and 235-236-CB) were produced from reductive dechlorination of higher chlorinated hepta-CB congeners (Figure S1 of the SI). Thus, the overall hexa-CB congeners, accounting for half of total PCBs in Aroclor 1260, were slightly decreased by 19.94% (Table S2 of the SI).

On the basis of appearance/disappearance of PCB congeners and their mass balance together with dechlorination of two PCB congeners (i.e., 2345-245-CB and 234-245-CB), dechlorination pathways were inferred for the dominant reductive dechlorination in culture AD14 (Figure S1 of the SI). Dechlorinators in culture AD14 primarily attacked flanked para-chlorines from 2345- and 245-chlorophenyl rings, and doubly flanked meta-chlorines from 2345- and 234-chlorophenyl rings. The dechlorination pattern mainly matches PCB dechlorination process H, which was first observed in situ both in the Acushnet Estuary (New Bedford, MA) and in parts of the Hudson River (New York).[34,35]

Microbial Community Structure in Culture AD14. To obtain a clear insight into microorganisms involved in reductive dechlorination of Aroclor 1260 in culture AD14, the whole microbial community structure was deciphered based on captured 34 724 pair-end Illumina sequences of the 16S rRNA genes (Figure 2). After 120 days of incubation, 6 genera of archaea and 15 genera of bacteria became dominant in the enrichment culture (≥0.5% in relative abundance for each genus). Among them, the high ratio of 16S rRNA gene sequences (45.92% of total sequences) of methanogens (e.g., Methanosarcina, Methanoculleus, and Methanosaeta) explained the abundant methane production (data not shown) in culture AD14. Two known dechlorinators were identified to be present in the sediment-free PCB dechlorinating culture, i.e., Dehalococcoides (2.12% of total sequences) and Dehalobacter (2.16% of total sequences). Illumina high throughput sequencing data ruled out the possibility that other known reductively dechlorinating bacteria (e.g., o-17/DF-1-type Chloroflexi, Desulfitobacterium, Geobacter, Sulfurospirillum, and Anaeromyxobacter) may be involved in dechlorination of Aroclor 1260 in culture AD14. Members of a new bacterial division, Acidobacterium (0.74% of total sequences), was also present in culture AD14 as shown in Figure 2. The rest mainly belonged to phyla of Firmicutes and Proteobacteria.

Reductive Dehalogenation of Other Halogenated Organic Compounds. To determine whether other common halogenated contaminants can be dehalogenated by culture AD14, four subcultures were set up with culture AD14 as inocula and with octa-BDE mixture (subculture AD14-PBDE), PCE (subculture AD14-PCE), 1,2-DCA (subculture AD14-DCA), and 2,4,6-TCP (subculture AD14-TCP), respectively, as an electron acceptor. After incubating for 75 days, subculture AD14-PBDE dechlorinated 24 and 173 nM of octa- and hepta-BDEs in the octa-BDE mixture (with an initial concentration of 27.12 nM nona-BDE, 124.01 nM octa-BDEs, 490.07 nM hepta-BDE, and 27.81 nM hexa-BDE) to the prominent tetra-BDEs (167.74 nM) (Figure 3A), and it dechlorinated TCE (~300 μM) • used for dissolving octa-BDE mixture powder • completely to ethene (data not shown). In both subcultures AD14-PCE and AD14-DCA, 675.4 μM PCE (Figure 3B) and 655.4 μM 1,2-DCA (Figure 3C) were completely dechlorinated to nontoxic ethene after 50 days and 35 days of incubation, respectively. Vinyl chloride (VC), the most toxic one among all chloroethenes, was not accumulated in subculture AD14-PCE, i.e., PCE was prominently dechlorinated to cis-DCE within the first 30 days, and further dechlorinated to ethene following another 20 days of incubation (Figure 3B). Subculture AD14-TCP can completely dechlorinate 2,4,6-TCP to 4-CP via 2,4-DCP within 10 days through removing ortho-chlorines (Figure 3D), and no further dechlorination to phenyl even after three extended months of incubation.

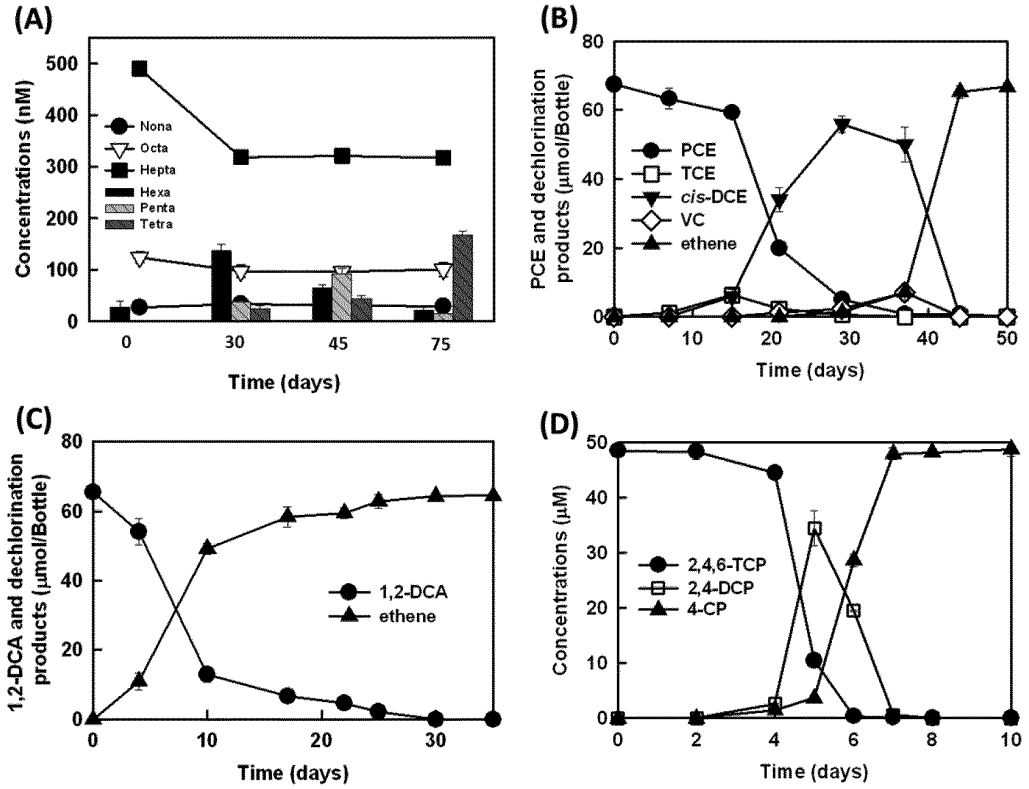

Figure 3. Dehalogenation of (A) octa-BDE mixture, (B) PCE, (C) 1,2-DCA, and (D) 2,4,6-TCP in AD14 subcultures.

In cultures amended with mixtures of the five halogenated compounds (i.e., 40.32 μM Aroclor 1260, 0.1 mM PCE, 0.6 mM 1,2-DCA, 10 μM 2,4,6-TCP, and 0.1 ppm octa-BDE mixture), complete dechlorination of 2,4,6-TCP (to 4-CP), 1,2-DCA (to ethene), and PCE (to ethene) were observed after incubating 14, 35, and 56 days, respectively. Tetra-BDEs as debromination products of octa-BDE mixture were detected on day 35, and the concentration further increased to 57.24 nM on day 56. For Aroclor 1260 dechlorination, similar PCB dechlorination products were observed after 4 months of incubation, which suggests no inhibition on PCB dechlorination even in the existence of other halogenated compounds (e.g., PBDEs, PCE, 1,2-DCA, and 2,4,6-TCP) in culture AD14.

Enrichment and Characterization of Dechlorinators. To enrich PCB dechlorinators present in culture AD14, alternative electron acceptors (i.e., octa-BDE mixture, PCE, 1,2-DCA, and 2,4,6-TCP) were used in order to speed up the process. After several serial transfers (11 times for subcultures AD14-PBDE, AD14-PCE, and AD14-DCA, and 25 times for subculture AD14-TCP), highly enriched subcultures were obtained and their community DNAs were extracted for subsequent DGGE analysis (Figure 4). Known dechlorinators enriched from these four subcultures were Dehalococcoides (present in subcultures AD14-PBDE, AD14-DCA, and AD14-PCE) and Dehalobacter (bacterium Deb-AD14-TCP from subculture AD14-TCP, and Deb-AD14-PCE from subculture AD14-PCE), which was consistent with the Illumina sequencing result of parent culture AD14.

Surprisingly, all Dehalococcoides mccartyi strains present in subcultures AD14-PBDE, AD14-DCA, and AD14-PCE shared identical 16S rRNA gene sequence over 520 bp (base 8-529, E. coli numbering), which was further confirmed by DGGE

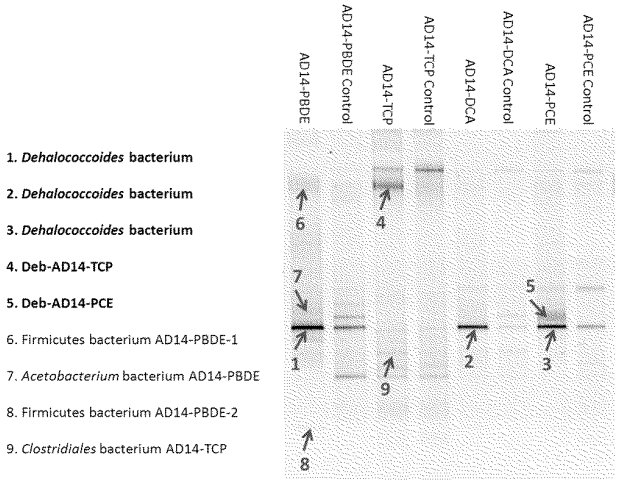

Figure 4. DGGE analysis of amplified 16S rRNA gene sequences from highly enriched subcultures and their controls (without halogenated compound amendment). Bands possibly correlated with dehalogenation activities were excised out for DNA extraction and subsequent sequencing. Obtained sequences were blasted and are shown on the left (known dechlorinators were highlighted with bold font).

analysis with Dehalococcoides genus-specific primers 1FGC/259R (Figure S2 of the SI). The nearly full-length 16S rRNA gene sequences of these dechlorinators were obtained by using 2S-DGGE method.[33]

Bacterium Deb-AD14-TCP identified in subculture AD14-TCP shared the highest 16S rRNA gene sequence identity (99% over 1421 bp) with Dehalobacter clone FTH2 (AB294743) from a 4,5,6,7-tetrachlorophthalide dechlorinating

culture (Figure S3 of the SI).[36] The closest relative of bacterium Deb-AD14-PCE enriched from subculture AD14-PCE was Dehalobacter sp. WL (DQ250129) by sharing 99% sequence identity over 1423 bp.

Dehalococcoides enriched in subcultures AD14-PBDE, AD14-PCE, and AD14-DCA share identical 16S rRNA gene sequences over 1353 bp, which have only 1 bp difference with that of their closest relative, Dehalococcoides mccartyi vs (CP001827) (Figure S3 of the SI). To further characterize the Dehalococcoides bacteria, PCR amplification with primers specifically targeting known functional genes (i.e., pceA, tceA, cbrA, vcrA, bvcA, and mbrA) was conducted, showing that only tceA (bacterium AD14-1) and vcrA (bacterium AD14-2) genes were present in these Dehalococcoides-containing subcultures (data not shown).

Quantification of PCB Dechlorinators. To determine whether dechlorinators enriched in these four subcultures still maintained PCB-dechlorination capabilities, Aroclor 1260 was amended to medium inoculated with subcultures pregrown on octa-BDE mixture, PCE, 1,2-DCA, or 2,4,6-TCP. After 90 days of incubation, only subculture AD14-PCE showed extensive dechlorination activity on Aroclor 1260, in which dechlorination products were the same with those formed in its parent culture AD14. Therefore, subculture AD14-PCE was reinoculated to Aroclor1260-spiked medium with acetate as a carbon source and H_2 as an electron donor, together with control bottles without Aroclor 1260 amendment, to quantify the growth of dechlorinators coupled with PCB dechlorination. As shown in Figure 5, both Dehalococcoides and Dehalobacter may

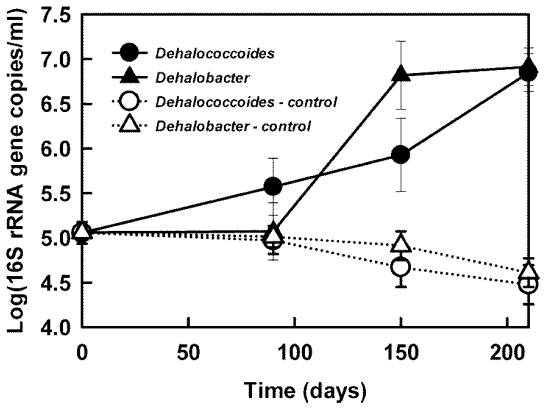

Figure 5. Growth of Dehalococcoides and Dehalobacter in culture AD14 and in its control bottles without PCBs amendment.

grow in PCBs-dechlorinating cultures. Compared with its parent culture AD14 amended with lactate or subculture AD14-PCE pregrown with PCE, the PCB dechlorination here had longer lag phase (i.e., ~90 days). Upon a decrease of average chlorine number per biphenyl from 6.38 to 5.87 (or 41.13 μM of total chlorine decrease, Figure S4 of the SI) after 210 days of incubation, 16S rRNA gene copy numbers of Dehalococcoides and Dehalobacter increased from 1.14×10^5 to 7.04×10^6 copies mL^{-1}, and from 1.15×10^5 to 8.20×10^6 copies ml^{-1}, respectively (Figure 5). Dehalobacter have a longer lag phase in growth, which might be due to lower bacterial activity or the requirement of intermediate PCBs generated by Dehalococcoides. Correspondingly, tceA and vcrA gene copy numbers increased from 5.40×10^4 to 2.73×10^6 copies mL^{-1}, and from 5.82×10^4 to 3.23×10^6 copies mL^{-1}, respectively, of which total gene copies were roughly equal to 16S rRNA gene copy numbers of Dehalococcoides. No growth of dechlorinators was observed in control bottles without Aroclor 1260 amendment.

Isolation of Two Dehalococcoides mccartyi Strains. To isolate tceA and vcrA gene-containing Dehalococcoides from subculture AD14-PCE, TCE, and VC were amended to their respective serial dilution bottles as sole electron acceptors. Since both strains can dechlorinate cis-DCE to VC, the culture of tceA gene-containing Dehalococcoides mccartyi strain AD14-1 was transferred to the next serial dilution bottles once observing 25−50% TCE being dechlorinated to cis-DCE. In following dilution series, TCE and VC dechlorination activities can be repeatedly observed in 10^{-7} TCE- and 10^{-6} VC-fed dilution vials, respectively. After 8 serial dilutions, uniform morphology of coccoid-shape bacteria through microscopy observation suggested that only Dehalococcoides mccartyi strains existed in these two dilution series (data not shown), which was verified by DGGE analysis (Figure S5A of the SI). Although similar to strain AD14-1 and strain AD14-2 sharing identical 16S rRNA gene sequences, Dehalococcoides mccartyi strain GT and strain CBDB1 can be differentiated by their adk and atpD genes (Figure S6 of the SI). Therefore, strain AD14-1 and strain AD14-2 were further distinguished by multilocus sequence typing (MLST) approach targeting these two housekeeping genes, which was developed specifically for unambiguous characterization of bacterial isolates.[37] Interestingly, the sequences of adk and atpD genes in strains AD14-1 and AD14-2 shared 100% sequence identities (over 464 bp for adk and 1201 bp for atpD). In addition, specific gene-targeted PCR showed culture AD14-1 (fed with TCE) and culture AD14-2 (fed with VC) possessing tceA and vcrA gene, respectively (Figure S5B of the SI). On the basis of the fact that common reductive dehalogenase (RDase) genes (e.g., tceA, vcrA, and bvcA)a res inglec opyg enesi nt he Dehalococcoides genomes,[38−41] RDase genes together with 16S rRNA genes can be monitored by qPCR to confirm the culture purity. This has been perfectly demonstrated in isolation of Dehalococcoides mccartyi strain GT.[41] To further corroborate the culture purity, qPCR analysis using universal and Dehalococcoides 16S rRNA gene- and RDase (i.e., tceA and vcrA) gene-targeted primers was performed in this study (Figure S7 of the SI). The total bacterial cell numbers in TCE- or VC-fed cultures almost equaled to the total Dehalococcoides cell numbers and tceA/vcrA gene copies, suggesting the purity of the cultures. The microscope observation, DGGE analysis, MLST result, and both the qualitative and quantitative PCR data all together verified the culture purity of strain AD14-1 and strain AD14-2.

Kinetic studies showed that strain AD14-1 can dechlorinate TCE (80.30 ± 3.28 umol/bottle) to VC (46.36 ± 1.33 umol/bottle) and ethene (32.83 ± 0.73 umol/bottle) within 30 days (Figure S8A of the SI), and strain AD14−2 dechlorinate VC completely to ethene after 20 day's incubation (Figure S8B of the SI). Strain AD14-1 and strain AD14-2 have distinct substrate range, as summarized in Table S3 of the SI. Among the potential electron acceptors tested, TCE, 1,2-DCA and octa-BDE mixture can be extensively dehalogenated by strain AD14-1, and DCEs, 1,2-DCA and 2,4,6-TCP can be dechlorinated by strain AD14−2. No Aroclor 1260 dechlorination activity was observed in either pure culture after 12 months of incubation, suggesting that these two isolates either incapable of dechlorinating PCBs or capable of dechlorinating PCBs but depending upon the presence of other beneficial bacteria. Neither of the two isolates showed fermentation

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capability on fumarate, malate, lactate, pyruvate, glucose, glumate, or used sulfate, sulfite, nitrate, or nitrite as an electron acceptor. Therefore, similar with other Dehalococcoides mccartyi strains, strain AD14-1 and AD14-2 requires hydrogen as an electron donor and acetate as a carbon source.

DISCUSSION

In this study, an enrichment culture AD14 was successfully established for extensive dehalogenation of Aroclor 1260 and the common coexisting halogenated compounds, e.g., PBDEs, PCE, 1,2-DCA, and 2,4,6-TCP. Although PBDEs were widely detected as cocontaminants at PCB contaminated sites,[4−6] no culture has been reported to dehalogenate both PCB and PBDE mixtures. PCB dechlorinators in culture AD14 are capable of extensively dehalogenating multiple compounds that are common contaminants in groundwater and sediments, e.g., PBDEs, 2,4,6-TCP, PCE, and 1,2-DCA. PCB dechlorination was not inhibited by the existence of above cocontaminated halogenated compounds because dechlorinators in culture AD14 could detoxify them more rapidly than could PCBs. Therefore, culture AD14 can be a special culture for cleaning up sites cocontaminated by PCB mixtures and other halogenated compounds (e.g., PBDEs and chloroethenes).

Both Dehalococcoides and Dehalobacter were identified in culture AD14 to be responsible for PCB dechlorination with evidence provided by both Illumina high-throughput sequencing and qPCR. The PCB dechlorinators identified in early studies were all placed within Chloroflexi phylum, i.e., Dehalococcoides[10,42] and o-17/DF-1 like bacteria.[10,12,43] The taxonomic identification of these PCB dechlorinators were usually conducted by DGGE[10,12,43] or PCR amplification with genus-specific primers.[42] Both methods have their own limitations in charactering PCB dechlorinating bacteria which are normally present as minor populations, e.g., DGGE can hardly discriminate bacteria with relative abundance less than 1% of total microbial community.[44] Compared with DGGE analysis, genus-specific PCR amplification is a faster and more sensitive way to identify PCB dechlorinators, especially when those bacteria exist as minor populations. However, this method may cause false negative results due to primer mismatch or limited primer coverage. Previous studies have shown that short reads from Illumina sequencing suffice for accurate microbial community analysis.[45] In this study, combination of Illumina high throughput sequencing, 2S-DGGE and qPCR analysis has been employed to pinpoint the PCB dechlorinating bacteria, an effective and comprehensive way to study microbial community structures.

Thus far, only two pure cultures (i.e., Dehalobium sp. DF-1 and Dehalococcoides mccartyi strain CBDB1) showed dechlorination activities on PCB mixtures, which were isolated on 2345-CB[14] and trichlorobenezes,[8] respectively. Isolation of PCB dechlorinators directly from Aroclor 1260-dechlorinating cultures is challenging due to their long lag phase and low growth rates on PCB mixtures.[46] Previous studies have shown that single dechlorinating bacteria can possess multiple RDase genes in single genomes,[38,40] and thus the same isolate may grow on various substrates.[11,18,40] Therefore, all of those observations suggest that PCB dechlorinators may be isolated by using alternative halogenated compounds. In this study, Dehalococcoides mccartyi strain AD14-1 and strain AD14-2 were isolated by using TCE or VC as an alternative electron acceptor rather than using PCBs. The two strains share identical 16S rRNA, adk, and atpD genes. However, both strains could not dechlorinate congeners in Aroclor1260 in defined medium amended with acetate and H_2 after 12 months of incubation. This might be attributed to the loss of the PCB dechlorinators or the functional reductive dehalogenase genes for PCB dechlorination during the isolation process, or PCB dechlorination requiring the cooperation of both Dehalobacter and Dehalococcoides. Another reason might be because the isolates require the existence of other beneficial bacteria to supply nutrients and cofactors. A similar phenomenon was observed in DF-1 pure culture, of which PCB dechlorination depended on the presence of cocultured Desulfovibrio species or its cell extract.[14] Whether strain AD14-1 or AD14-2 is able to dechlorinate PCBs still warrants future studies. However, isolation of both strains in this study may shed light on future cultivation of other PCB dechlorinators.

In conclusion, an enrichment culture AD14 was developed to dehalogenate PCBs and other halogenated compounds (i.e., PBDEs, PCE, 1,2-DCA, and 2,4,6-TCP). The Dehalobacter species was confirmed to grow in a PCBs-dechlorinating culture. In addition, two Dehalococcoides mccartyi strains with identical 16S rRNA gene sequences were isolated to be able to dechlorinate TCE and VC.

ASSOCIATED CONTENT

Supporting Information

The primer sequences used in this study (Table S1); inferred PCB dechlorination pathways and PCB homolog distribution in AD14 culture (Figure S1 and Table S2); various incubation and dechlorination paradigms (Figures S2−S8); and strain AD14-1 and strain AD14-2 distinct substrate ranges (Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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